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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/IS95/00001 <b>(22) International Filing Date:</b> 3 November 1995 (03.11.95) <b>(30) Priority Data:</b> 4231 4 November 1994 (04.11.94) IS <b>(71) Applicant (for all designated States except US):</b> GENIS HF. [IS/IS]; Efna- og líftækniúts, Keldnaholt, IS-112 Reykjavík (IS). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> THORBJARNARDOTTIR, Sigríður [IS/IS]; Laboratory of Molecular Genetics, Institute of Biology, University of Iceland, IS-108 Reykjavík (IS). JONSSON, Zophanias, O. [IS/CH]; Institut für Veterinärbiochemie, Universität Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich (CH). PALSDOTTIR, Astríður [IS/IS]; Institute for Experimental Pathology, University of Iceland, Keldur, Vesturlandsveg, IS-128 Reykjavík (IS). EGGERTSSON, Guðmundur [IS/IS]; Laboratory of Molecular Genetics, Institute of Biology, University of Iceland, IS-108 Reykjavík (IS). KRISTJANSSON, Jakob, K. [IS/IS]; Technological Institute of Iceland, Keldnaholt, IS-112 Reykjavík (IS).		<b>(74) Common Representative:</b> GENIS HF.; Efna- Og líftækniúts, Keldnaholt, IS-112 Reykjavík (IS). <b>(81) Designated States:</b> AU, CA, CN, FI, IS, JP, KR, NO, NZ, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). <b>Published</b> With international search report.
<b>(54) Title:</b> A GENE FROM THE BACTERIUM RHODOTHERMUS MARINUS, CODING FOR THE AMINO ACID SEQUENCE OF DNA-LIGASE, PRODUCED IN ESCHERICHIA COLI OR SOME OTHER SUITABLE HOST ORGANISM <b>(57) Abstract</b> <p>This invention provides a new DNA ligase enzyme, derived from the thermophilic bacterium <i>Rhodothermus marinus</i>, active at temperatures between 5-75 °C with half life of 7 min. at 90 °C. The invention constitutes the nucleotide sequence of the gene, coding for the enzyme, its expression in a productive host, purification of the protein to near homogeneity and the use of the enzyme in cycle sequencing. Unique properties of this enzyme make it suitable for use in the SPEL-6 method of primer walking (sequential primer elongation by ligation of 6-mers) initially described by Szybalski (5) and computer-controlled automatic sequencing of long DNA sequences.</p>		

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**A gene from the bacterium *Rhodothermus marinus*, coding for the amino acid sequence of DNA-ligase, produced in *Escherichia coli* or some other suitable host organism.**

## 5 FIELD OF THE INVENTION

This invention relates to a gene originating from *Rhodothermus marinus* and the encoded thermostable DNA ligase enzyme, active over a wide range of temperatures (5-75°C), a unique property which allows the enzyme to be used in cycle sequencing of long DNA sequences, using computer-controlled automatic equipment. The enzyme can ligate small oligomers during  
10 repeated thermal cycles, producing suitable primers for sequencing. This eliminates the need for time consuming subcloning procedures and primer synthesis.

## BACKGROUND OF THE INVENTION

DNA ligases catalyze the formation of phosphodiester linkages between adjacent 5'-phosphoryl  
5 and 3'-hydroxyl groups in double-stranded DNA. The reaction proceeds in three steps, initiated by the formation of a stable enzyme-(lysine-e)-adenylate complex (for review see Lehmann, 1974) (1).

Various analytical methods are based on the use of thermostable ligases. The detection of single base mutations or specific nt sequences by ligase chain reaction (LCR) (2) is well documented  
20 as is the use of repeat expansion detection (RED) for the detection of trinucleotide repeats (3). Of considerable potential is the use of thermostable DNA ligase in localized DNA detection by circularization of oligodeoxynucleotides as described by Nilson et al. (4) and to construct sequencing primers from hexameric nucleotides as proposed by Szybalski (5) and Kaczorowski (6). This method has not yet been fully applicable, since present commercially available DNA  
25 ligases, do not possess the dual property of being active at low temperatures and stable at high temperatures. The invention of such enzyme, would radically change the concept of automatic sequencing of long DNA sequences, such as entire genes or genomes.

In search of thermostable DNA ligases, active over a wide range of temperatures, we have cloned the ligase gene of the thermophilic eubacterium, *Rhodothermus marinus* (7). *R. marinus*  
30 was originally isolated from a marine alkaline hot spring in Iceland (8) and deposited in "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Macheroder Weg 1b, D-38124 Braunschweig/Germany" (strain number: DSM 4253). Analysis of 16S rRNA places it close to the root of the Flexibacter-Cytophaga-Bacteroides group (9).

## 35 SUMMARY OF THE INVENTION

This invention provides a new DNA ligase enzyme, active at temperatures between 5-75°C with half life of 7 min. at 90°C. The invention constitutes the nucleotide sequence of the gene, coding for the enzyme, its expression in a productive host and purification of the protein to near  
homogeneity.

40 The properties of this enzyme make it suitable for use in the SPEL-6 method of primer walking (sequential primer elongation by ligation of 6-mers) initially described by Szybalski (5) and computer-controlled automatic sequencing of long DNA sequences.

## DESCRIPTION OF THE FIGURES

Fig. 1.

Sequence of the *R. marinus* ligase gene. The sequence is given in lowercase letters and the deduced amino acid sequence of the protein is given in capital letters aligned with corresponding codons. Restriction sites for NcoI (CCATGG) and NdeI (CATATG) are underlined. A potential ribosomal binding site ACCAGAG 6 bp upstream of the Met codon is also underlined. The stop codon is marked with an asterisk.

Fig. 2.

Denaturation curves of three thermostable ligases at 91°C. a: *R. marinus* ligase. b: *T. scotoductus* ligase (solid curve) and *T. thermophilus* ligase (dashed curve). The curves were drawn using exponential regression. Vertical bars indicate the standard deviations of the measurements.

Fig. 3

The *R. marinus* and *T. scotoductus* DNA ligase enzymes were set to react on nicked pUC19 plasmid DNA at different temperatures, and the DNA was analysed by agarose gel electrophoresis. On gel A and B, the arrows point to different forms of plasmid DNA. Arrow I indicates supercoiled monomer (form I), II relaxed open circular monomer (form II) and III linear plasmid (form III). The ligated supercoiled plasmid (form I) migrates faster than the relaxed circular and linear DNA (form II and III) in EtdBr containing agarose gels.

20 Gel-A: Temperature dependency of nick closing activity

The incubation temperatures for 2.5 fmol of *R. marinus* were as follows: lane 1, 5°C; lane 2, 15°C; lane 3, 25°C; lane 4, 35°C; lane 5, 45°C; lane 6, 55°C; lane 7, 65°C; and lane 8, 75°C.

25 The incubation temperatures for 4 fmol of *T. scotoductus* ligase were as follows: lane 9, 5°C; lane 10, 15°C; lane 11, 25°C; lane 12, 35°C; lane 13, 45°C; lane 14, 55°C; lane 15, 65°C; and lane 16, 75°C.

Lane A represents 400 ng of pUC19 plasmid DNA, and lane B represents approximately 400 ng of nicked pUC19 plasmid DNA.

Gel B: Titrations of nick closing activity at different temperatures

30 Lanes 1-8 represent *R. marinus* ligase incubated with nicked pUC19 DNA at 5°C (lanes 1-4) and at 15°C (lanes 5-8). Lane 1 contains 5 fmol ligase; lane 2 contains 10 fmol ligase; lane 3 contains 20 fmol ligase; lane 4 contains 40 fmol ligase; lane 5 contains 5 fmol ligase; lane 6 contains 10 fmol ligase; lane 7 contains 20 fmol ligase, and lane 8 contains 40 fmol ligase.

35 Lanes 9-16 represent *T. scotoductus* ligase incubated with nicked pUC19 DNA at 35°C (lanes 9-12) and 45°C (lanes 13-16). Lane 9 contains 8 fmol ligase; lane 10 contains 16 fmol ligase; lane 11 contains 32 fmol ligase; lane 12 contains 64 fmol ligase; lane 13 contains 8 fmol ligase; lane 14 contains 16 fmol ligase; lane 15 contains 32 fmol ligase, and lane 16 contains 64 fmol ligase.

40 Lane A represents 400 ng pUC19 plasmid DNA, and lane B represents approximately 400 ng nicked pUC19 plasmid DNA.

## DETAILED DESCRIPTION OF THE INVENTION

### Cloning:

The ligase encoding gene was cloned into *E. coli* by complementation of a temperature sensitive (ts) mutation. Gene libraries were made by ligating Sau3AI partially digested genomic DNA from the thermophilic bacterium into BamHI digested pUC18 plasmids. Plasmids complementing the ts mutation were selected and the presence of the ligase genes confirmed by complementation of two additional ts ligase mutations. Further proof was obtained by assaying nick-closing enzyme activity in heat treated crude extracts.

### Sequencing:

The cloned gene was subcloned into M13mp18/19 and completely sequenced in both directions (Fig. 1). The sequence information was used to clone the gene into the high expression vector pET23, using PCR and restriction enzymes. For expression we used an *E. coli* strain carrying a ts ligase mutation and chromosomally inserted DE3 prophage. The prophage contains a T7 polymerase gene under the control of an IPTG inducible lac promoter. This setup secures high expression of ligase. After induction with IPTG for 4 hours, ligase can make up as much as 15% of total cell protein.

### Enzyme Purification:

The enzyme has been purified to near homogeneity using a simple method relying on heating at 80°C for 30 minutes to denature most of the proteins produced by the host, DEAE-sepharose chromatography to remove contaminating nucleic acids, a Cibacron Blue column for further purification and gel filtration for desalting.

### Enzyme characteristics:

A novel assay for ligase activity, used for measuring DNA ligase thermostability was applied on the *R. marinus* enzyme and two other reference enzymes (*Thermus thermophilus* (Stratagene, La Jolla, CA, USA) and *Thermus scotoductus* (our own clone)) at 91°C (Figs. 2a and 2b). Dilutions of the enzymes were incubated at 91°C in ligase reaction buffer and aliquots taken at regular intervals. The aliquots were added to a reaction mix containing two differentially end labeled primers and the complementary template. After thermal cycling, the amount of ligated doubly labeled product was assayed with ELISA. The half life of the *R. marinus* enzyme was estimated to be 7 min. (Fig. 2a) but both the *T. thermophilus* and *T. scotoductus* ligases are somewhat more heat stable, their half lives being around 26 min. at 91°C (Fig. 2b). Our estimate of the half life of *T. thermophilus* ligase is in agreement with the half life of 30 min. at 90°C reported by the manufacturer.

Under our standard assay conditions for enzyme activity measurements on agarose gels (Fig. 3A and 3B), nick closing activity (10) was detected after 15 min. incubation with 20 fmol of *R. marinus* ligase at 5°C, whereas *T. scotoductus* ligase activity could not be detected below 25°C under the same conditions. However, at lower concentrations (approx. 2.5 fmol/l), ligation was not detected with the *R. marinus* ligase at 5°C. The *R. marinus* ligase is suitable for the SPEL-6 method of primer walking (6) and permits even cycling as in cycle sequencing (Szybalski and Kaczorowski, personal communication).

The SPEL abbreviation stands for "Sequential Primer Elongation by Ligation". The SPEL-6 method of primer walking, described by Szybalski (5), represents an original strategy of using hexameric DNA of different sequences for production of primers that can be used in DNA

sequencing procedures. By combining three hexamers of selected contiguous nucleotide sequences, in a ligation reaction, the SPEL method provides a fast and simple way of constructing primers for sequencing, and eliminates the need for synthesis of individual primers.

5 The key to the SPEL-6 method of primer walking, is a DNA ligase, sufficiently active at low temperatures for ligation of the selected complimentary hexameres, but stable enough to survive subsequent thermal dissociation of produced primers from the template DNA. Consequently, the *R. marinus* ligase can ideally be used in computer-controlled automatic cycle sequencing, incorporating a ligation step in each cycle.

10 The SPEL-6 method of primer walking eliminates the need for subcloning, permits direct sequencing of large DNA fragments, is ideally suited for automation, and should accelerate the sequencing of large genomes by more than one order of magnitude (6).

#### **Enzyme Thermostability:**

15 Dilutions of the *R. marinus* and *T. scotoductus* ligases were incubated at 91°C and samples containing approximately 40 ng (~ 500 fmol) protein were taken at regular intervals. A novel assay based on the ligation of two end-labelled 25-nt oligos paired to the complementary single-stranded template was used to measure the activity of the heat-treated ligase. The template used was a part of the *R. marinus* 16S rRNA gene cloned into M13mp19. Two adjacent 25-nt oligos complementary to a 50 bp motif in the chosen template, were obtained from Operon Technologies. The 3' end of the oligo complementary to the 5' site of the template was labeled with biotin and the 5' end phosphorylated (pCCAGGGCTICACACGTGCTACAATG-Bio). The other oligo complementary to the 3' site of the 50-nt template, was 5' labeled with fluorescein (Flu) (Flu-CGTCAAGTCATCATGGCCCTTACGC). The heat-treated ligases were mixed with 230 fmol template and 10 pmol of each 25-nt primer in 40 µl of standard assay buffer (see section (d)) and overlaid with paraffin oil. The reactions were cycled 20 times 25 between 90°C and 60°C in a thermal cycler and kept for 20 sec at each temperature. Triplicate aliquots of 5 µl from each reaction were then applied to wells in streptavidin coated 96 well microtiter plates (Nunc MaxiSorp) with 45 µl washing buffer (100 mM Tris-HCl pH 8/150 mM NaCl/0.05% Tween 20). The plates were incubated for 30 min. at 37°C, washed twice with denaturation buffer (10 mM NaOH/0.05% Tween 20), once more with washing buffer and then 30 incubated for 30 min. at 37°C with 50 µl of a 1:2000 diluted anti-fluorescein-alkaline-phosphatase conjugate (Boehringer Mannheim) in washing buffer. After washing six times with washing buffer, 100 µl of fresh substrate solution (1 mg per ml pNPP in 10% diethanolamine-HCl pH 9.8/0.56 mM MgCl<sub>2</sub>) were added. The resulting yellow color was read with a microplate reader (Fig. 2).

#### **35 Enzyme Activity At Different Temperatures:**

pUC19 DNA was nicked with DNaseI as described by Takahasi et al. (1984). 400 ng nicked DNA was incubated with ligase at various temperatures (5-75°C) for 15 minutes in 15 µl reaction mix containing 20 mM Tris-HCl pH 8, 75 µM NAD<sup>+</sup>, 10 mM MgCl<sub>2</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 100 g/ml BSA (acetylated). The reaction mixtures were electrophoresed 40 immediately on 0.7 % agarose gel containing 50 µg/l EtdBr. The band pattern in each lane, indicate occurrence of ligation (Fig. 3).

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**CLAIMS**

1. An isolated DNA which codes for a recombinant thermostable DNA-ligase and is obtainable from *Rhodothermus marinus*, consisting essentially of the nucleotide sequence shown in Fig. 1.
- 5 2. DNA sequence according to claim 1, coding for a DNA-ligase which is active in DNA-ligation, within a temperature range of about 5 to 75°C.
3. DNA sequence according to claim 1 or 2, coding for DNA-ligase which is active in DNA-ligation, at temperatures below 25°C.
4. DNA sequence according to claim 1, 2 or 3, coding for DNA-ligase, having a half life of about 7 minutes at 91°C.
- 10 5. A cloning vector comprising one of the isolated DNA of claim 1 to 4.
6. A host organism of cell transformed by the vector of claim 5.
7. A recombinant thermostable DNA-ligase being active in DNA-ligation, within a temperature range about 5 to 75°C.
- 15 8. The recombinant thermostable enzyme of claim 7, expressed by the nucleotide sequence shown in Fig. 1 and obtained from *Rhodothermus marinus*.
9. The recombinant thermostable enzyme of claim 7 or 8, characterized by a half life of about 7 minutes at 91°C.
- 10 10. Use of the enzyme according to one of the claims 7 to 9 in the SPEL-6 method of primer walking and cycle sequencing.
- 20





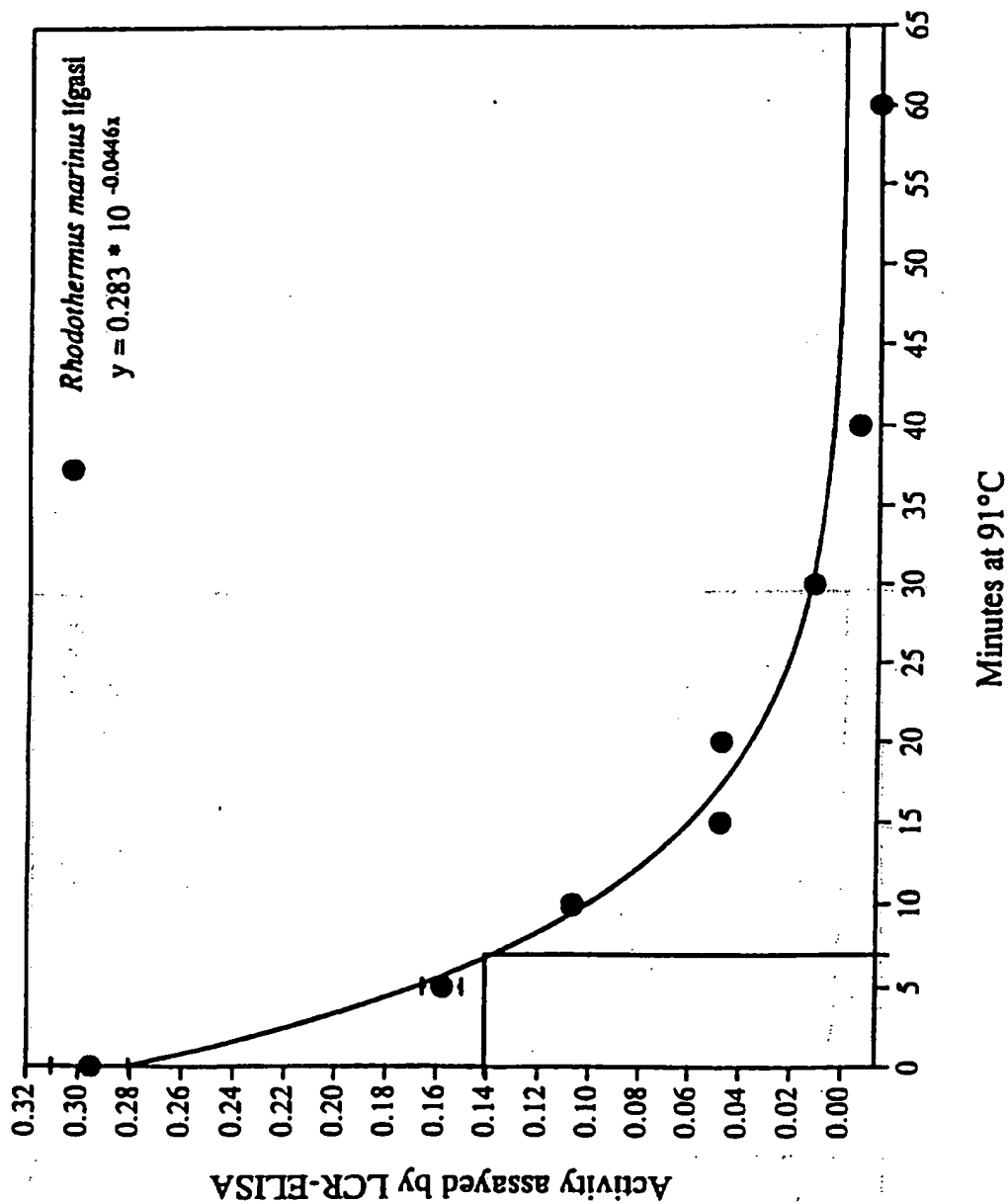


Fig. 2a

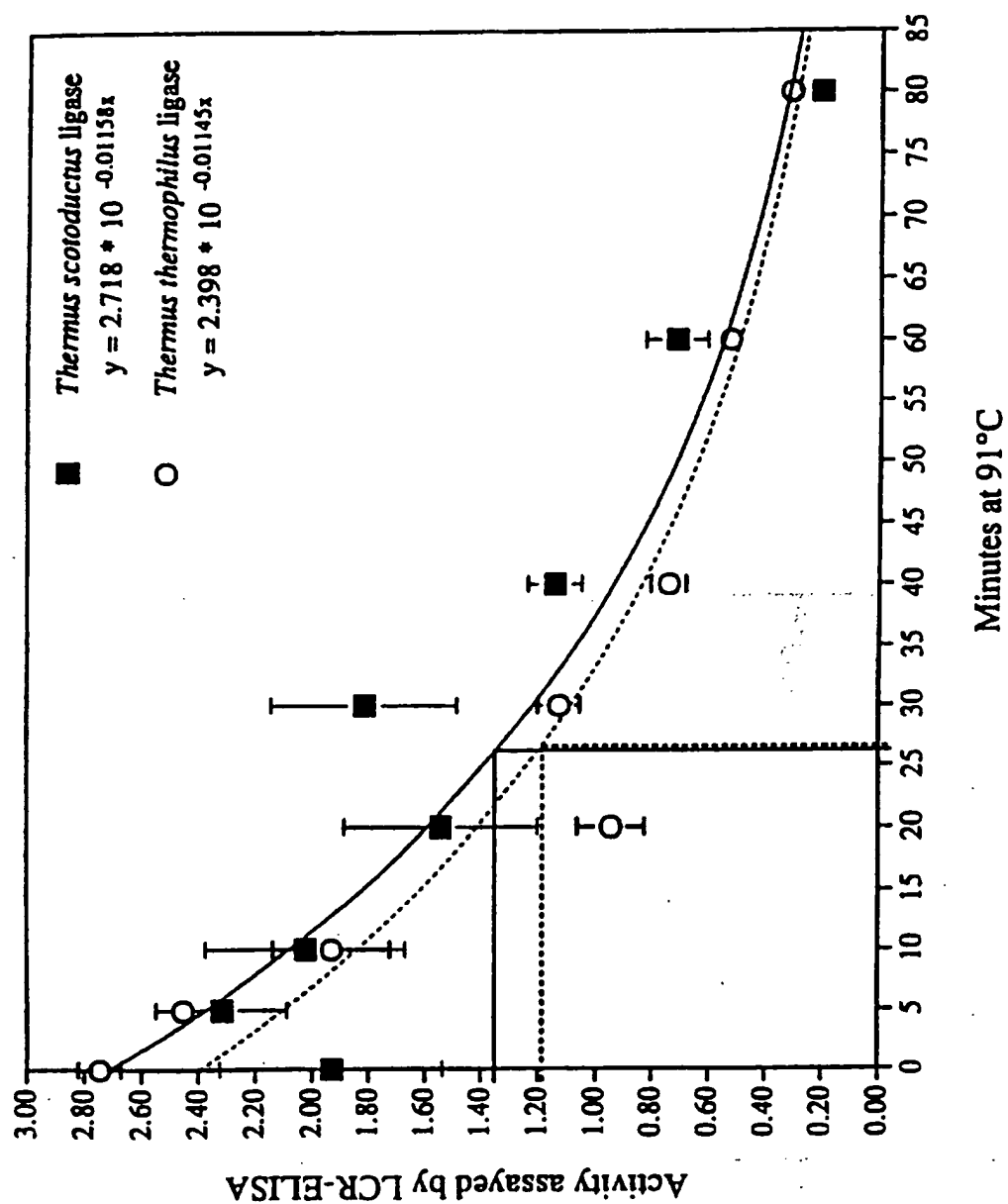


Fig. 2b

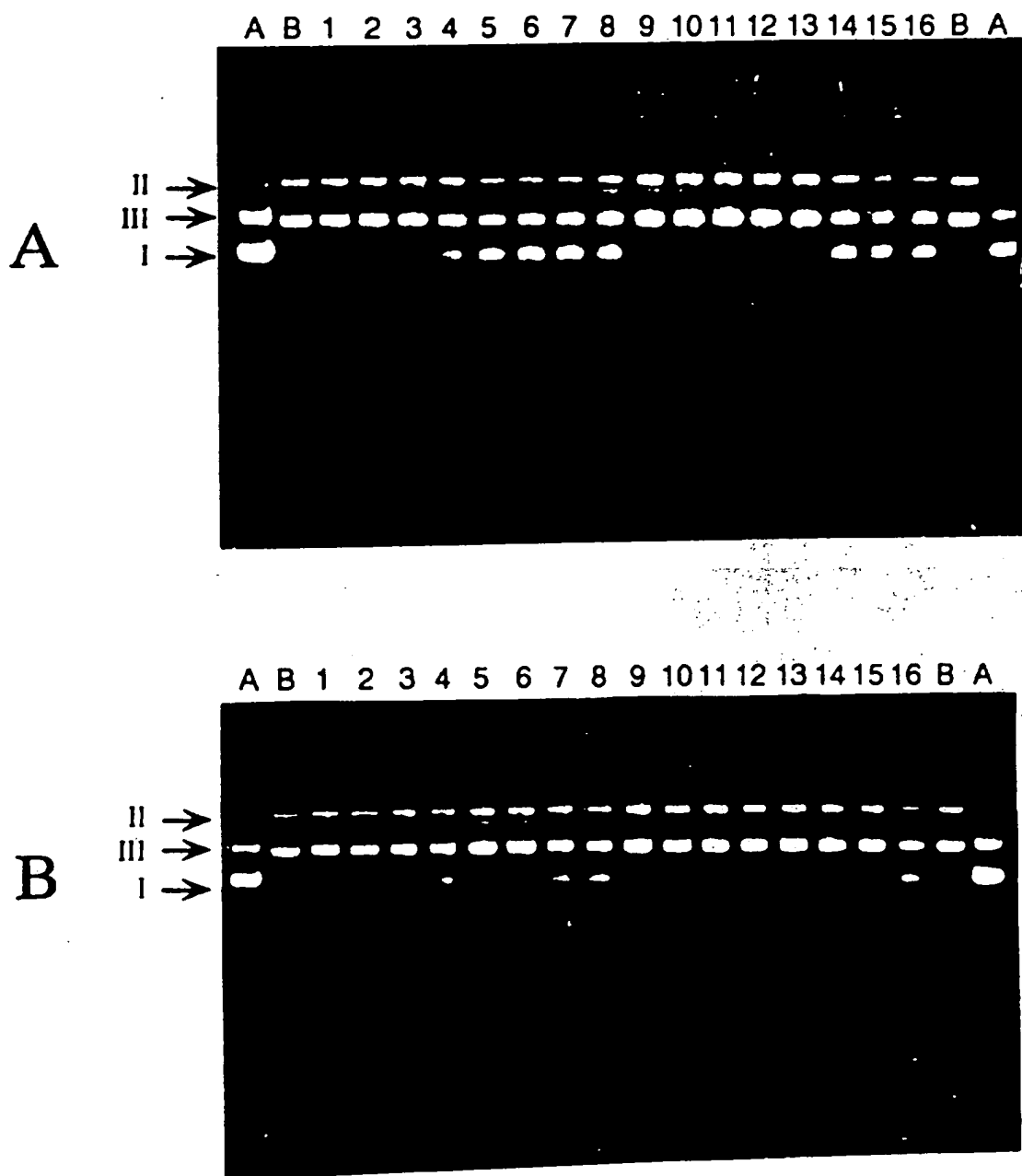


Fig. 3

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**INTERNATIONAL SEARCH REPORT**

International application No.

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**A. CLASSIFICATION OF SUBJECT MATTER**

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**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Chemical Abstracts, Volume 123, No 25, 18 December 1995 (18.12.95), (Columbus, Ohio, USA), Thorbjarnardottir, Sigridur. H. et al, "Cloning and sequence analysis of the DNA ligase-encoding gene of Rhodothermus marinus, and overproduction, purification and characterization of two thermophilic DNA ligases", page 487, THE ABSTRACT No 333386x, Gene 1995, 161 (1), 1-6  --	1-10
X	Chemical Abstracts, Volume 101, No 17, 22 October 1984 (22.10.84), (Columbus, Ohio, USA), Takahasi, Miho et al, "Thermophilic DNA ligase. Purification and properties of the enzyme from Thermus thermophilus HB8", page 292, THE ABSTRACT No 146594x, J. Biol. Chem. 1984, 259 (16), 10041-10047  --	1-10



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2  
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9402615 A1 (STRATAGENE), 3 February 1994 (03.02.94), see page, line 24-28 and page 5, line 30-34 - page 6, line 1-3 and fig 3  --	1-10
X	Patent Abstracts of Japan, Vol 9, No 96, C-278, abstract of JP, A, 59-227295 (KAGAKU GIJIYUTSUCHIYOU), 20 December 1984 (20.12.84)  --	1-10
A	Chemical Abstracts, Volume 117, No 3, 20 July 1992 (20.07.92), (Columbus, Ohio, USA), Lauer, Gail et al, "Cloning, nucleotide sequence, and engineered expression of Thermus thermophilus DNA ligase, a homolog of Escherichia coli DNA ligase", page 189, THE ABSTRACT No 21385d, J. Bacteriol. 1991, 173 (16), 5047-5053  --	1-10
A	WO 9117239 A1 (CORNELL RESEARCH FOUNDATION INC.), 14 November 1991 (14.11.91), see page 16, line 13-16  --	1-10
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A	Chemical Abstracts, Volume 118, No 13, 29 January 1993 (29.01.93), (Columbus, Ohio, USA), Barany, Francis et al, "Cloning, overexpression and nucleotide sequence of a thermostable DNA ligase-encoding gene", page 353, THE ABSTRACT No 119741n, Gene 1991, 109 (1), 1-11  --	1-10

## INTERNATIONAL SEARCH REPORT

International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Chemical Abstracts, Volume 108, No 17, 25 April 1988 (25.04.88), (Columbus, Ohio, USA), Alfredsson, Gudni A. et al, "Rhodothermus marinus, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland", page 428, THE ABSTRACT No 147065g, J. Gen. Microbiol. 1988, 134 (2), 299-306</p> <p style="text-align: center;">-- -----</p>	1-10

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

05/02/96

International application No.

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9402615	03/02/94	NONE	
WO-A1- 9117239	14/11/91	EP-A, A- 0528882	03/03/93
EP-A1- 0571880	01/12/93	NONE	